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Identification of resistance-associated proteins in closely-related maize lines varying in aflatoxin accumulation

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Abstract *Aspergillus flavus* infection of maize and subsequent contamination with carcinogenic aflatoxins poses serious health concerns, especially in developing countries. Maize lines resistant to *A. flavus* infection have been identified; however, the development of commercially-useful aflatoxin-resistant maize lines has been hindered due to a lack of breeding markers. To identify maize resistance-associated proteins (RAPs) as potential markers for breeding, 52 BC1S4 lines developed from crosses between five African maize inbreds and five temperate aflatoxin-resistant lines were screened using the kernel screening assay. Five pairs of closely-related lines that had 75–94% genetic similarity within each pair and which varied within each pair in aflatoxin accumulation were selected for proteomic investigation. Kernel embryo and endosperm protein profile differences within the pair and across pairs were compared using two-dimensional polyacrylamide gel electrophoresis. Differentially expressed (≥1.5-fold) RAPs were sequenced through tandem mass spectrometry and were identified as antifungal, stress-related, storage or regulatory proteins. Sequence homology analysis highlighted several proteins in maize that confer resistance to *A. flavus* infection and/or aflatoxin production.

Keywords Two-dimensional gel electrophoresis · Corn · Protein profile comparison · Aflatoxin resistance · Antifungal protein · Stress-related protein

Abbreviations

2D	Two-dimensional
COR	Cold regulated
CV	Coefficient of variation
ESI–MS/MS	Electron spray ionization tandem mass spectrometry
EST	Expressed sequence tag
GLX	Glyoxalase
HSP	Heat-shock protein
IITA	International Institute of Tropical Agriculture
KSA	Kernel screening assay
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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PAGE	Polyacrylamide gel electrophoresis
RAPs	Resistance associated proteins

Introduction

Aflatoxin contamination of maize (*Zea mays* L.) after infection by *Aspergillus flavus* is a serious agricultural problem, especially under hot and dry conditions (Diener et al. 1987; Payne 1998), and a frequent occurrence in the southern USA and parts of Africa. Aflatoxins, the toxic and highly carcinogenic secondary metabolites produced by the fungus, significantly reduce the value of grain both as an animal feed and as an export commodity (Nichols 1983). They also pose health hazards to humans (Hsieh 1989) and to domestic animals (Smith and Moss 1985). Monitoring and strict regulation of aflatoxins in food and feed by the US Food and Drug Administration (FDA) offers a strong measure of protection to consumers. However, the situation in vulnerable African nations is not nearly as positive. This was highlighted by a study that revealed a strong association between exposure to aflatoxin and both stunting (a reflection of chronic malnutrition) and being underweight (a reflection of acute malnutrition) in West African children (Gong et al. 2002), and also by a 2004 outbreak of acute aflatoxicosis in Kenya caused by the ingestion of contaminated maize and resulting in 125 deaths (Probst et al. 2004).

Maize becomes contaminated with aflatoxins prior to harvest (Lillehoj 1987). Therefore, the discovery of maize genotypes having natural resistance to *A. flavus* infection and aflatoxin accumulation (King and Scott 1982; Gardner et al. 1987; Widstrom et al. 1987; Scott and Zummo 1988; Brown et al. 1995; Campbell and White 1995) has enhanced efforts to employ a host resistance strategy to combat this problem. However, breeding markers are needed in order to transfer resistance alleles from resistant germplasm to elite commercial backgrounds. Proteome investigations comparing domestic resistant and susceptible maize lines have identified a number of constitutively-produced resistance-associated proteins (RAPs) from both kernel embryo and endosperm tissue (Chen et al. 2002, 2007). An earlier study demonstrated that constitutively-produced proteins play a critical role in conferring resistance to aflatoxin production (Chen et al. 2001). These proteins can be grouped into three categories based on peptide sequence homologies: storage proteins, stress-related proteins, and antifungal or potentially antifungal proteins (Chen et al. 2002, 2007).

This identification of RAPs in domestic lines required the initial development of composite resistant and susceptible protein profiles constructed based on the analysis of gels of a number of genotypes. A simpler approach, when possible, is to use maize lines with close genetic backgrounds which differ in aflatoxin resistance. To develop maize inbred lines with enhanced resistance to aflatoxin contamination in good agronomic backgrounds, a USA–Africa collaboration was established (Menkir et al. 2006, 2008). Several US maize lines with proven resistance to aflatoxin accumulation (Brown et al. 1995; Campbell et al. 1997) were crossed to five elite tropical inbred lines from the International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeria. The five African lines originally selected for resistance to ear rot caused by *Aspergillus, Botrydiplodia, Diplodia, Fusarium*, and/or *Macropomina*, under severe disease pressure in Central and West Africa, demonstrated potential aflatoxin-resistance using the kernel screening assay (KSA) (Brown et al. 2001; Menkir et al. 2006). The resulting F1 crosses and backcross populations (BC1) were self-pollinated to develop inbred lines with resistance to aflatoxin production (Menkir et al. 2006). All lines at each generation were selected for foliar disease resistance and for good agronomic characteristics, through the S4 generation. This resulted in the development of 144 S4 lines and 65 BC1S4 lines (Menkir et al. 2006).

In the present study, fifty-two of the 65 BC1S4 lines with 75–94% common genetic background were screened for aflatoxin accumulation using the KSA. Five pairs of these closely-related lines significantly different in aflatoxin accumulation within the pair were subjected to proteomic analysis to identify variations in protein production between resistant and susceptible lines. Success in this type of investigation could lead to the identification of markers for transferring resistance. A preliminary report has been published (Chen et al. 2005).

Materials and methods

Breeding closely-related maize lines

Seven genotypes from the USA (B73, GT-MAS:gk, Mo17, Mp420, Oh516, T115, and Tex6) (Brown et al. 1995; Campbell et al. 1997) were crossed to five African tropical elite maize inbred lines (Babangoyo, Ku1414SR, 1368, 4001, and 9450) at IITA in Ibadan, Nigeria in 1999. A backcross (BC1) was made to each of the F1 crosses using the respective genotype from the USA as a recurrent parent during the 2000 dry season and was self-pollinated thereafter. From the 2000 rainy season, ear-to-row selection was made from each backcross population to develop inbred lines at IITA. At each generation of inbreeding, visual selection within and among lines was made on the basis of synchrony between pollen shed and silking, low ear placement, well-filled ears, and resistance to lodging and diseases, including *Puccinia polysora* rust, *Bipolaris maydis* blight, and *Curvularia lunata* leaf spot. This was done under naturally occurring disease pressure in Ibadan.

Determining aflatoxin resistance of closely-related lines

Sixty-five BC1S4 lines shared at least 75% common genetic background, and were thus defined as genetically closely-related lines. These lines were planted at Saminaka (8°39′E, 10°34′N, altitude 760 m) in Nigeria in two rows of 5 m length, with a spacing of 0.75 m between rows and 0.50 m between plants within a row. Saminaka has been used as a suitable seed production site by breeders because the location receives sufficient precipitation every year and pressure from major lowland diseases is minimal. Fifty-two of these lines, which produced a sufficient amount of kernels, were subjected to aflatoxin screening under laboratory conditions in six separate groups using the KSA (Brown et al. 1995). MI82, a resistant inbred obtained from the Department of Crop Sciences of the University of Illinois-Urbana, and P3142, a susceptible line obtained from Pioneer Hi-Bred International (Johnston, IA, USA), were included as controls in each group. After inoculation with *A. flavus* strain AF13 (ATCC 96044; SRRC1273) and 7 days of incubation using the KSA protocol, kernels were dried and aflatoxins were extracted and analyzed (Brown et al. 1995). This experiment was conducted twice and aflatoxin data from the two experiments which were significantly different were not combined, notwithstanding the consistent ranking of the lines within each group. Data from one experiment are presented in this study.

Embryo and endosperm separation and protein extraction

Kernels (20 g) from each of five pairs of closely-related lines varying in aflatoxin resistance were dissected into embryo and endosperm after soaking in water overnight at 0°C. These conditions were employed to facilitate the separation without protein induction (confirmed using Western blot analysis). Embryo and endosperm proteins were extracted as previously described by Chen et al. (2002, 2007) and desalted before resolubilizing in lysis buffer (Görg et al. 1998) at a final concentration of 2 μg/μl for analytical or 20 μg/μl for preparative gels.

Two-dimensional gel electrophoresis and gel analysis

Fifty (analytical) or 700 μg (preparative) of embryo or endosperm protein was applied to rehydrated 17-cm Immobiline DryStrip gels (pH 3–10). One- and two-dimensional (2D) gel electrophoresis was performed as described by Chen et al. (2002). Protein spots in analytical gels were stained automatically with a Silver Stain Kit (Genomic Solutions, Chelmsford, MA, USA) using an Investigator Gel Processor (Genomic Solutions). Preparative gels were stained with Coomassie Brilliant Blue R 250. All stained gels were scanned using a UMAX PowerLook II scanner (UMAX data systems, Taiwan), and analyzed using the Progenesis SameSpot software package (Nonlinear Dynamic, Durham, NC, USA). Only the reproducible spots were used for comparison (over 90% of all protein spots detected on 2D gels for a given genotype were reproducible). This experiment was performed twice, and each sample was run in triplicate.

The normalized spot volume, which was adjusted for loading and staining variations between gels, was used for protein level comparison. Technical variations were determined using protein samples extracted from the same genotype at three different times and separated on the same set of gels. The

coefficient of variation (CV) of normalized volume for matched protein spots ranged from 6 to 17%. The CV value of normalized volume was slightly higher (from 7 to 20%) when three protein extracts of the same genotype were separated on three separate runs, which were similar to what was reported by Fuxius et al. (2008). Based on this, protein spots showing at least 1.5-fold differences in normalized volume between two genotypes were considered differentially expressed. A comparison was made to identify unique or 1.5-fold up/down-regulated spots, first within each pair, then across pairs. For endosperm samples, only three pairs had enough proteins for proteomic analysis. All differentially expressed proteins that were identified were sequenced as described below.

Peptide sequencing and database sequence homology analysis

Protein spots of interest were recovered from preparative 2D gels, subjected to in-gel trypsin digestion, and sequenced at Baylor College of Medicine (Houston, TX, USA) as previously described (Chen et al. 2002). The MS-Fit program (http://prospector.ucsf.edu/) was used to search the spectra from matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) against the National Center for Biotechnology Information (NCBI) non-redundant protein databases (version 20091202) and translated expressed sequence tag (EST) databases. The parameters used for database searches were: maximum number of missed cleavages: 2; peptide N terminus: hydrogen; peptide C terminus: free acid; cysteine modification: acrylamide; minimum matches: 4; considered modifications: peptide N-terminal Gln to pyroGlu, oxidation of methionine and protein N-terminal acetylation; minimum parent ion matches: 1; and MS/MS fragment tolerance ± 0.2 Da. Probability-based Mowse scores were used to define the quality of identification. The threshold value (80) was calculated based on a 5% possibility of false identification. If a positive protein identification was not made, de novo peptide sequences were obtained using a PE SCIEX API 3000 (Applied Biosystems, Foster City, CA, USA) electro-spray ionization tandem mass spectrometer (ESI–MS/MS) equipped with a Protana nanospray source (Odense, Denmark). Peptide sequence homology searches were performed using BLAST (Altschul et al. 1997) against known proteins or translated open reading frames of ESTs in databases at NCBI and SWISS-Prot. The mapping information of RAP genes was obtained by searching their corresponding DNA sequences against Maize GenomeBrowser (http://archive.maizesequence.org) and Maize Genetics and Genomics Database (http://www.maizegdb.org/).

Statistical analysis

All data were analyzed using the analysis of variance procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Aflatoxin data were log-transformed prior to analysis to equalize variances. Means were separated by Duncan's multiple range test ($P \leq 0.05$).

Results

Breeding closely-related lines differing in aflatoxin resistance

Fifty-two of the 65 BC1S4 lines were screened using the KSA in six separate groups to determine their aflatoxin resistance. The parental backgrounds of these 52 BC1S4 breeding lines are summarized in Electronic Supplementary Material Table S1. The mean aflatoxin produced in each line is summarized in Table 1. Most of these lines supported aflatoxin levels higher than that of the resistant control MI82. However, some of the lines had resistance levels close to or better than the resistant control, MI82, such as lines 23, 25, 31–33, 36, 41, 42, 47, and 50–53 (Table 1). Several of these lines also supported significantly lower aflatoxin production than some of their sister lines developed from the same backcross. Five pairs of lines from five different backcrosses with contrasting levels of resistance to aflatoxin production within the pair and with a sufficient amount of kernels were selected for further analysis through proteomics (Table S2). Their genetic background similarity within the pair ranged from 75 to 94% (Table S2).

Table 1 Aflatoxin production in kernels of the 52 BC1S4 maize breeding lines

Experiment no. 1		Experiment no. 2		Experiment no. 3	
Line[x]	Toxin, ppb[y]	Line	Toxin, ppb	Line	Toxin, ppb
54	5,966 a	Suscept.cont.	10,197 a	T115	7,854 a
59	3,005 b	22	1,693 b	16	3,959 b
GT-MAS:gk[z]	2,663 b	19	1,284 bc	12	2,292 bc
29	2,511 b	28	1,605 bcd	18	1,641 bcd
57	1,620 bc	21	1,072 bcd	13	782 bcd
54	1,295 bcd	27	1,025 bcd	14	458 cd
30	1,022 cd	26	793 bcde	Suscept.cont.	438 cd
Suscept. cont.	1,189 cd	20	574 cde	17	378 cd
58	1,952 cde	24	399 cde	15	302 cd
33	612 de	GT-MAS:gk	338 de	52	220 d
31	475 de	25	228 e	53	232 d
32	449 de	23	197 e	Resist.cont.	147 d
55	348 de	Resist.cont.	76 e		
Resist.cont.	6 e				
Experiment no. 4		Experiment no. 5		Experiment no. 6	
Line[x]	Toxin, ppb[y]	Line	Toxin, ppb	Line	Toxin, ppb
39	1,594 a	Suscept.cont.	5,064 a	Oh516	5,214 a
37	1,674 a	10	4,021 a	Suscept.cont.	3,098 a
Suscept.cont.[z]	2,109 a	48	1,797 b	11	2,202 a
Mp420	1,464 a	49	1,158 bc	63	1,687 a
61	1,370 a	Resist.cont.	879 bc	34	1,191 b
60	1,449 ab	46	515 bc	35	374 c
43	772 ab	51	640 bc	Resist.cont.	0 d
38	1,249 abc	45	375 bc		
44	793 abc	47	568 c		
40	636 bcd	50	358 c		
62	299 cde				
36	63 def				
41	320 efg				
42	43 fg				
Resist.cont.	110 g				

[x] See Table S1 for pedigrees of these 52 lines

[y] Data were transformed using $\log(y + 1)$ before statistical analysis to equalize variations. Within the same column, the aflatoxin values followed by the same letter(s) do not differ significantly based on Duncan's test; *ppb*, parts per billion

[z] The resistance (MI82) and susceptible (Pioneer 3154) maize controls (*resist.cont, suscept.cont.*) used in this KSA assay were grown in the US. The other US resistant lines (GT-MAS:gk, Mp420, T115, and Oh516) were grown in IITA, as were the rest of the crosses

Identification of differentially expressed proteins in the embryo and endosperm

A comparison of embryo proteins between the five pairs showed that 18 protein spots were uniquely or differentially expressed in the resistant lines compared to their corresponding susceptible lines. These proteins were separated into three groups based on their patterns of expression (Table S3). Several proteins were found to be significantly up- or

down-regulated (≥1.5-fold) in at least three resistant lines, such as spots 337, 436, 473, 492, 535, 546, 547, and 564 (Table S3). Six other proteins were differentially expressed in two or three pairs, but were either missing or did not change significantly in the remaining pairs, such as 490, 494, 514, 518, 537, and 567 (Table S3). There were also some spots (384, 1058, 1073, and 1540) that were unique or differentially expressed only in one pair, but were missing or remained the same between resistant and susceptible lines in the remaining pairs (Table S3). Examples of these differentially expressed embryo proteins (spots 490, 492, and 518) in resistant lines are shown in Fig. 1.

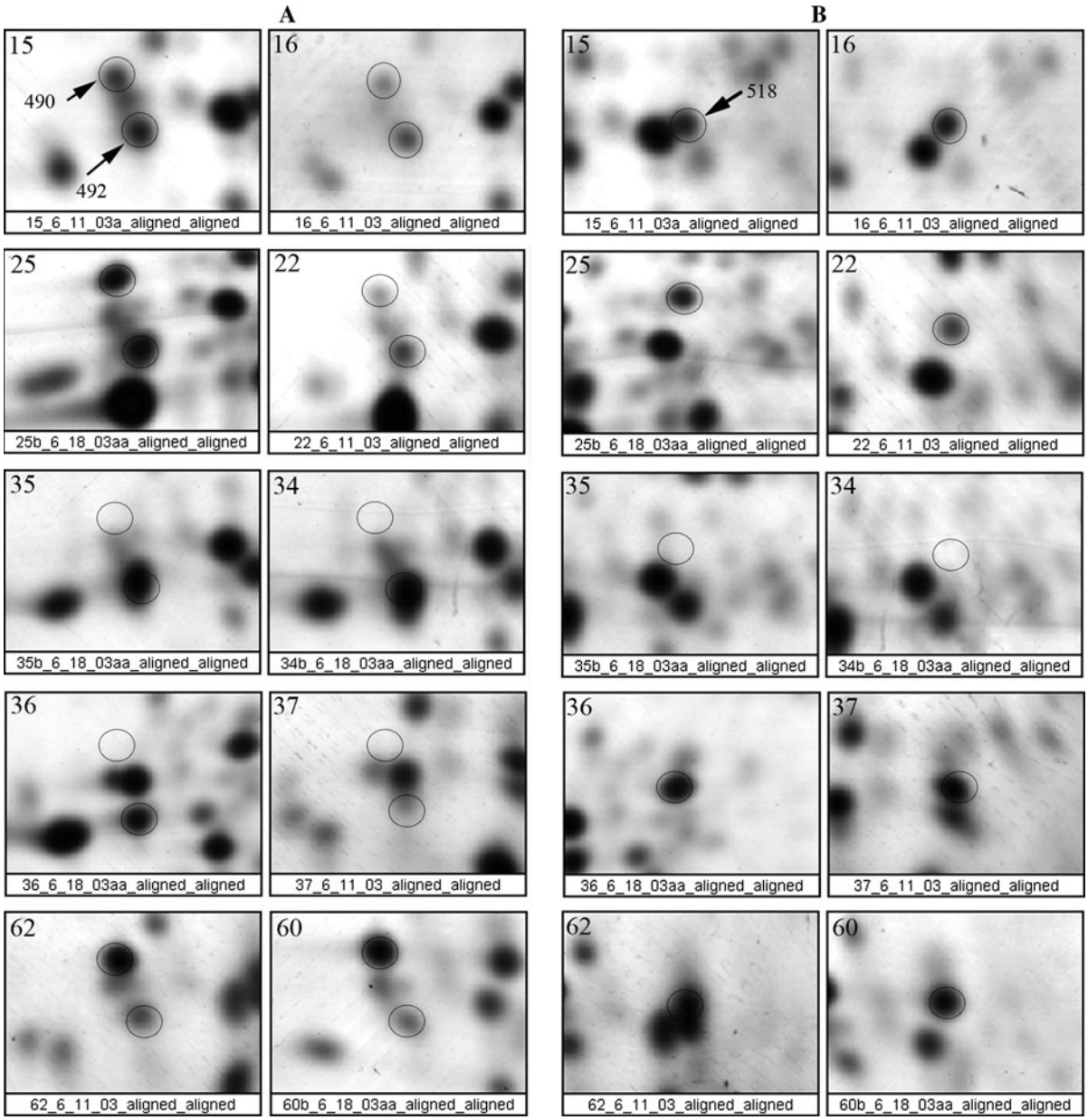

Fig. 1 Protein spots differentially expressed in the embryo tissue between resistant (on the *left*) and susceptible (on the *right*) closely-related maize lines. **a**, spots 490 and 492, which were up-regulated in the resistant line of two and three pairs, respectively; **b**, spot 518, which was up-regulated in the resistant line of two pairs, but was missing or did not change significantly in the other three pairs

The proteins extracted from endosperm tissue also were compared to identify protein differences. Twelve protein spots uniquely or differentially expressed in the resistant lines were identified and separated into three groups based on their pattern of expression (Table S3). The first group consists of two up-regulated protein spots (992 and 1068) found in the resistant lines in all three pairs. The majority of differentially expressed proteins, such as spots 815, 945, 1057, 1180, 1229, and 1521, form the second group that show differential expression in two of the three resistant lines (Table S3). The third group consists of the remaining four spots, 842, 1356, 1369, and 3443, that were only differentially expressed in a resistant line of one pair, but were either missing or did not change significantly in the other two pairs (Table S3). Examples of these differentially expressed endosperm proteins (spots 992, 1057, and 1356) in the resistant lines are shown in Fig. S1.

Identification of the unique and differentially expressed protein spots using MALDI-TOF

Twenty-eight differentially expressed spots were recovered (16 from embryo and 12 from endosperm), digested in-gel with trypsin and analyzed first using MALDI-TOF. Six spots were positively identified through peptide mass fingerprinting. Two spots (494 and 514) from embryo and two (815 and 1369) from endosperm were identified as maize globulin-2 precursor protein with 7–12 masses matched and a coverage ranging from 18 to 25%. The Mowse scores for the matches ranged from 2102 to 3.09E + 05 (Table 2). The spectrum analysis also indicated that the methionine residues at several positions were oxidized. Four other peptide masses from spot 1369 matched to the previously reported 14-kDa trypsin inhibitor antifungal protein (Chen et al. 1998), indicating that this spot contains at least two different

Table 2 The identity and chromosome location of the differentially expressed proteins identified through MALDI-TOF

Spot no.	Tissue	Mowse score[a]	No. of masses matched[b]	% Coverage	Accession no.	Protein name	Modifications	Virtual bins[c]
494	Emb	3.09E + 05	10/26	23	1802402A	Globulin-2 precursor	M-ox at 120,143	1.11
514	Emb	2.75E + 05	12/35	25	1802402A	Globulin-2 precursor	M-ox at 92, 120, 143	1.11
815	Endo	2,688	7/59	18	1802402A	Globulin-2 precursor	ND	1.11
1057	Endo	1,832	7/22	36	P41980, AAA72022	Maize Mn-superoxide dismutase	ND	8.03
1068	Endo	2.06E + 06	13/33	58.0	P33679 P23867,	Zeamatin/22 kDa alpha-amylase/ trypsin inhibitor antifungal protein	PyroGlu at 143/144.	7.04
1369	Endo	2,102	9/27	23	1802402A	Globulin-2 precursor	M-ox at 143	1.11
		126	4/27	25	X54064	Bifunctional Hageman factor/alpha amylase inhibitor	ND	2.06
1521	Endo	1.61E + 04	10/29	71	P46517, S16249	Late embryo-genesis abundant protein	M-ox at 60	6.05

MALDI-TOF search parameters: maximum number of missed cleavages: 2; peptide N terminus: hydrogen; peptide C terminus: free acid; cysteine modification: acrylamide; minimum matches: 4; considered modifications: peptide N-terminal Gln to pyroGlu, oxidation of methionine (M-ox) and protein N-terminal acetylation; and minimum parent ion matches: 1; MS/MS fragment tolerance: ±0.2 Da; *ND*, not detected

[a] MS-Fit program (http://prospector.ucsf.edu/) was used to search the MS spectra from MALDI-TOF against NCBI non-redundant protein databases (version 20091202) and translated EST databases. A Mowse score of 80 or higher was considered significant. This threshold value was calculated based on a 5% possibility of false identification

[b] Number of peptide masses out of the total number of peptide masses that match the calculated masses of the target protein

[c] The mapping information of RAP genes was obtained by searching the sequences from the *underlined* accessions against Maize Genome Browser (http://archive.maizesequence.org) and Maize Genetics and Genomics Database (http://www.maizegdb.org/). Virtual bins are computational representations of genetic bins found on the maize genetic maps

proteins. Seven out of the 22 peptide masses of spot 1057 from endosperm matched a maize manganese-superoxide dismutase (P41980) with a 36% coverage and a Mowse score of 2688 (Table 2). Thirteen peptide masses from spot 1068 with 58% coverage matched a maize 22-kDa zeamatin/trypsin inhibitor protein with a Mowse score of over 2.0E + 06 (Table 2). The spectrum analysis also indicated that the Glu residue at position 143/144 of this 22-kDa protein was modified (Table 2). Ten out of 29 peptide masses from spot 1521 covering 71% of the whole sequence matched a late-embryogenesis-abundant protein with a significantly high Mowse score of 1.6E + 04. The chromosomal locations of the corresponding genes of these identified proteins in the maize genome were also mapped by searches against the recently completed maize genome sequences (http://archive.maizesequence.org) and (http://www.maizegdb.org/) and are listed in Table 2. The MS-Fit search of peptide masses of the remaining 14 spots from embryo and seven from endosperm did not yield significant matches.

Peptide sequencing, homology analysis, and the chromosomal locations of the unique and differentially expressed protein spots

The peptides that could not be positively identified through MALDI-TOF were further analyzed using ESI–MS/MS to obtain de novo peptide sequences (Table 3). These proteins were then identified based on homology analysis of the peptide sequences obtained in this manner. Peptide sequences obtained from spots 436 and 473 of embryo were almost identical, and overlapped the sequences obtained from spot 945 from the endosperm (Table 3). Their peptide sequences showed more than 98% identity to maize ESTs that have significant homology to cupin-domain-containing proteins (Fig. S2a). Spot 473 could be a breakdown product of spot 436, since both have the same pI, but different molecular mass. The peptide sequences from spot 1058 and 1073 were identical, and matched a cupin family protein from maize (ACG25229). However, they shared little sequence similarity to those of spot 436/473 and did not appear to share the same immediate ancestral gene (Fig. S2b).

Peptide sequences of six spots (490, 492, 518, 547, 564, and 1540) from the embryo and one spot (1180) from endosperm showed high homology to 16.9- to 18-kDa small heat-shock families of proteins from maize and other plant species (Fig. 2a). The peptide sequences from spots 492 and 564 showed high homology to a 17.2-kDa heat-shock protein (HSP) (X65725), whereas sequences from spot 1540, which overlapped with those from spot 547, showed a 96% identity to the deduced amino acid sequence of a maize EST (BE639130) with a calculated molecular mass of 16.8 kDa (Fig. 2a). The peptide sequences from spot 490, which overlapped with the three peptides from spot 1180 and the one from spot 518, completely matched to the deduced amino acid sequences of a different maize EST (AW258080), a 17.9-kDa HSP from pearl millet (X94193), and an 18.0-kDa HSP from rice (U83670). The relative similarities of these small HSPs are depicted in Fig. 2b, which indicates the presence of at least three different families of HSPs in maize, with spots 518 and 1180 forming one family (17.9-kDa HSP), spots 547 and 1540 forming the second family (16.7-kDa HSP), and spots 492 and 564 forming the third family (17. 2-kDa HSP). The remaining partial sequences of spot 518 showed high homology to a cold regulated (COR) protein from *Hordeum vulgare* (AJ291295), *Triticum aestivum* Wcor18 (AB097412), and an EST from *Zea mays* (DQ245527) (Table 3). The other peptide from spot 1180 showed a complete match to a maize translation initiation factor 5A (P80639 and Y07920) (Table 3).

The peptide sequences of spots 384 and 546 from embryo both showed significant homology to glyoxalase I (Table 3). The peptide sequences from spot 546 showed 100% identity to a glyoxalase family protein (ACG23936) from maize (Fig. S3), high homology to a glyoxalase I family protein from rice (ABF95269) and a putative receptor serine/threonine kinase from *Arabidopsis* (AAG48811). However, this protein is different from spot 384 and the previously identified glyoxalase I from maize (AY241545) or rice (AB017042) (Fig. S3), which share over 95–100% sequence identity to each other. Two other spots (337 and 537) from the embryo were identified as an embryo-specific protein of unknown function and a glucose/sorbitol dehydrogenase (Table 3), respectively.

In the endosperm, the peptide sequence of spot 842 showed a high homology to β-1,3-glucanases from common wheat (*Triticum aestivum,* AAY88778),

Table 3 Peptide sequences obtained through ESI–MS/MS, their identities and their location in the maize genome

Spot no.	Tissue	Peptide sequences	Identity or homology	% Identity	Scores	E value	Virtual bins[a]
337	Embryo	EVDLPASTTAGAGR GGVLFMPGVPGVVER TIHFWQVDR	An embryo-specific protein of unknown function (AI676923, AF049892 and AY106294)	100	47.8	2E–06	4.06
384	Embryo	VVLVDNTDFLK IASFVDPDGWK QPGPLPGINTK SAEAVDLATK ADTPEPLCQVMLR YTIAMLGYADEDK	Maize glyoxalase I protein (BT016228, AY241545, AY106316)	100	105	3E–21	10.03
436/ 473[b, c]	Embryo	IYAIFTSEGINADDPSKPK LGFGVQ**P**EVVEAIK TPPPIIAYNPEEK VEAYSSVSNLVK GFETDVLR	Cupin-domain containing protein, vicilin storage protein (BT024037, CD443502) (1 mismatch)	98.5	125	4E–27	9.07
490[c]	Embryo	SLIR(the S is acetylated) VEVEDGNVLQISGER AAME**D**GVLTVTVPK	Maize HSP17.2/17.9 (AW258080, DQ244781, AAPO6883) (1 mismatch)	96	42.4	7E–5	1.03
492[c]	Embryo	SIVPSATSTNSETAAFASAR AGLE**D**GVLTVTVPK	Maize HSP17.2 (X65725, AY109481, DQ244240) (1 mismatch)	97.1	39.7	0.26	3.04
518	Embryo	VEVEDGNVLQISGER	Maize HSP16.9/17.2/17.9 (CO520475, AW258080)	100	32.7	0.068	1.03
		….I/LGGI/LQ/KE….. K/RPEGF/M(ox)SGAAGAQ/KQ/KGR …AI/LDDS….	Maize cold regulated protein (COR18) (DQ245527, AB097412)	88.0	31.5	0.43	8.04
537	Embryo	EPMALPADLGYEANCR IDVVVNNAAEQYER ESIGDVTEADLER	Glucose and ribitol dehydrogenase, and short-chain dehydrogenases, dormancy related protein (DQ244551, AW257889, T06212)	100	81.3	1E–16	2.08
546	Embryo	AASFYDAAFGYTVR AVDDGAVAVSAPEDKPWGQK ETDELSGAVQLPDSSAAGR DMDGNIVR	Glyoxalase family protein, (ACG23936, AY105485, DQ245596)	100	91.3	7E–17	1.03

Table 3 continued

Spot no.	Tissue	Peptide sequences	Identity or homology	% Identity	Scores	E value	Virtual bins[a]
547[c]	Embryo	SETAAFASAR VEVEDGNVL**I/L**ISGQR AALE**D**GVLTVTVPK	Maize HSP17.2 (BE639130, AY109481, DQ244240) (2 mismatches)	92.3	38.9	0.46	3.04
564[c]	Embryo	SIVPSA**V**STNSETAAFASAR VEVEDGNVLVISGQR AGLE**D**GVLTVTVPK	Maize HSP17.2 (X65725, DQ244240, AY109481) (2 mismatches)	95.9	57	2E–06	3.04
1058/ 1073	Embryo	FLGGAGDPASVIAGFGPK	Match to a cupin family protein from maize (ACG25229)	100	56.2	4E–07	1.04
1540[c]	Embryo	VEVEDGNVLLISGQR AALE**D**GVLTVTVPK SIVPSS**P**SSAAASETAAFASAR	Maize HSP16.9/17.2 (DQ244781, and BE639130) (2 mismatches)	96.0	41.6	2E–04	3.04
842	Endo	YVAVG NEVQGDDTR DISLGY ATFQPGTTVR	Beta-1, 3-glucanase from rye (ACF83600, AM181314)	100	59.2	3E–09	3.05
945[c]	Endo	VEAYSSVSN(I/L) VK (F/ox-M) TSEG(I/L)NA…(K/Q) PK	A cupin family protein, vicilin storage protein or globulin-like protein (BT024037, CD444191, CD443502)	100	39.2	0.13	9.07
992	Endo	VTFPIIADPVK LSFLYPATTGR	1-cys peroxiredoxin antioxidant (PER1) (DQ378060, DQ244712, BT016686)	100	49.8	9E–05	7.05
1180	Endo	LPTDETLVAQIK	Eukaryotic translation initiation factor 5A from maize (T01355, AF034943, P80639, Y07920)	100	40.5	0.056	7.04
		SLIR TSSETAAFAGAR VEVEDGNVLQISGER	Heat shock protein 17.9 (AW258080, DQ244781)	100	75.3	2E–12	1.03
1356	Endo	MEQTFIMIKPDGVQR	Nucleoside diphosphate kinase from Arabidopsis (NM_117000, AY104578, S31446);	100	54.9	3E–06	7.03
		VFLAGTNSALQK LLAFGADEEQQVDR	Maize globulin-2 (X53715, AY104085, X59085, S15675)	100	76.6	8E–13	1.11

Table 3 continued

Spot no.	Tissue	Peptide sequences	Identity or homology	% Identity	Scores	E value	Virtual bins[a]
3443[c]	Endo	PLNADAIR EAAPAAECCAGVK (D)(V)SCADVDAN(S)(R)(P)CVGYVTGK	Putative lipid transfer protein from *Sorghum bicolor* (AF466200, AAL73541, CO465387, ES702554) (2 mismatches)	95.6	61.3	3E–08	10.03

[a] The mapping information of RAP genes was obtained by searching the sequences from the *underlined* accessions against Maize Genome Browser (http://archive.maizesequence.org/) and Maize Genetics and Genomics Database (http://www.maizegdb.org/). Virtual bins are computational representations of genetic bins found on the maize genetic maps

[b] The only difference in peptide sequences between spots 436 and 473 is that spot 436 lacks the first two amino acid residues (*underlined*) of spot 473

[c] Amino acid residues in *bold* did not match to the sequence in the databases; amino acid residues separated by a "/", such as I/L, Q/K, and F/ox-M indicate that either amino acid could be true based on MS/MS; amino acid residues in *parentheses* indicate those residues were not certain

barley (*Hordeum vulgare,* P15737), and rye (*Secale cereale,* AM181314) (Fig. 3). It also completely matched an unknown protein (ACF83600) and the deduced amino acid sequences of several ESTs (EC888785, EE166887) from maize, indicating that this is an uncharacterized maize glucanase (Fig. 3). The two peptide sequences from spot 992 matched completely to peroxiredoxin antioxidant (PER1). Spot 1356 appears to contain two proteins. One of the peptide sequences (MEQTFIMIKPDGVQR) matched a nucleoside-diphosphate kinase from *Arabidopsis thaliana* (S31446) and the deduced amino acid sequences of a maize EST (AY10578) (Table 3). The remaining two peptides, however, matched a maize globulin-2 precursor (X53715, AY104085). Peptide sequences from spot 3443 showed significant homology to a putative lipid transfer protein from *Sorghum bicolor* (AF466200) (Table 3). The tentative genome locations of these RAP genes were also determined by searching corresponding accessions in Maize Genome Browser (http://archive.maizesequence.org/index.html) and Maize Genetics and Genomics Database (http://www.maizegdb.org/) (Table 3). For the remaining three protein spots (535 and 567 from embryo, 1229 from endosperm), no peptide sequences were obtained due to poor digestion.

Discussion

Breeding for resistance to *A. flavus* infection/aflatoxin contamination in maize has been a slow process in the two decades since the discovery of natural resistance in maize. In the present study, 52 BC1S4 lines, derived from crosses involving US aflatoxin-resistant lines and African lines originally selected for resistance to ear rot under severe disease pressure in Central and West Africa, were screened for aflatoxin accumulation. Approximately half of the collection of original lines selected in Africa for ear rot resistance had shown low levels of aflatoxins when screened with the KSA (Brown et al. 2001). That ear rot resistance and aflatoxin resistance were not correlated in more of these lines may be due to the fact that maize ear rot disease is also caused by *Botrydiplodia, Diplodia, Fusarium*, and/or *Macropomina* (Menkir et al. 2006; Balint-Kurti and Johal 2009).

Although markers are readily available for maize breeding (Coe et al. 2002; Mammadov et al. 2010;

A

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Spot518/1180      -----SLIR-------------------------------------TSSETAAFAGAR- 16
Pg_X94193         ----MSLIRRSNVFDPFSLDLWDPFEGFPFGSGSNSG-SLFPSFPR-TSSETAAFAGARI 54
Zm_AW258080       ----MSLIRRSNVFDPFSLDLWDPFEGFPFGSGSSS--SLFPSFPR-TSSETAAFAGARI 53
Os_U83670         ----MSLIRRSNVFDPFSLDLWDPFDGFPFGSGSRSSGTIFPSFPRGTSSETAAFAGARI 56
Spot490           ----------------------------------------------TSSETAAFAGAR- 12
Spot492           ------------------------------SIVPSA---------TSTNSETAAFASAR- 20
Zm_X65725         ----MSLVRRSNVFDPFSMDLWDPFDTMFRSIVPSA---------TSTNSETAAFASARI 47
Spot564           ------------------------------SIVPSA---------VSTNSETAAFASARV 21
Spot547/1540      ------------------------------SIVPSSP-------SSAAASETAAFASAR- 22
Zm_BE639130       NEAEMSLVRRSSVFDPFSVDLFDPFDSMFRSIVPSS--------SSAAASETAAFASARI 52
                                                              *******.**

Spot518/1180      ------------------------VEVEDGNVLQISGER---------------------- 31
Pg_X94193         DWKETPEAHVFKADVPALKKEEVKVEVEDGNVLQISGERNKEQE-EKTDTWHRVERSSGK 113
Zm_AW258080       DWKETPEAHVFKADVPGLKKEEVKVEVEDGNVLQISGERNKEQE-EKTDTWHRVERSSGR 112
Os_U83670         DWKETP-EHVFKADVPGLKKEEVKVEVEDGNVLQISGERSKEQE-EKTDKWHRVERSSGK 114
Spot490           ------------------------VEVEDGNVLQISGER---------------------- 27
Spot492           ------------------------------------------------------------
Zm_X65725         DWKETPEAHVFKADLPGVKKEEVKVEVEDGNVLVISGQRSREKE-DKDDKWHRVERSSGQ 106
Spot564           EVEDG------------------------------------------------------- 26
Spot547/1540      ------------------------VEVEDGNVLLISGQR---------------------- 37
Zm_BE639130       DWKETPEAHVFKADLPGVKKEEVKVEVEDGNVLLISGQRSRXRRRTXGDKWHRVERSSGQ 112
                                          *********.***.*

Spot518/1180      ----------------------------------------------
Pg_X94193         FMRRFRLPENAKTDQIRASMENGVLTVTVPKEEVKKPEVKSIQISG 159
Zm_AW258080       FLRRFRLPENAKTEQIRAAMENGVLTVTVPKEDVKKPEVK------ 152
Os_U83670         FLRRFRLPENTKPEQIKASMENGVLTVTVPKEEPKKPDVKSIQVTG 160
Spot490           ------------------AAMEDGVLTVTVPK--------------- 41
Spot492           ------------------AGLEDGVLTVTVPK--------------- 34
Zm_X65725         FIRRFRLPDDAKVDQVKAGLENGVLTVTVPKAEEKKPEVKAIEISG 152
Spot564           --------NVLVISGQRAGLEDGVLTVTVPK--------------- 49
Spot547/1540      ------------------AALEDGVLTVTVPK--------------- 51
Zm_BE639130       FVRRFRLPENAKTEEVRAALENGVLTVTVPKAEVKKPEVKSIQIS- 157
                                  *..*.*********
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B

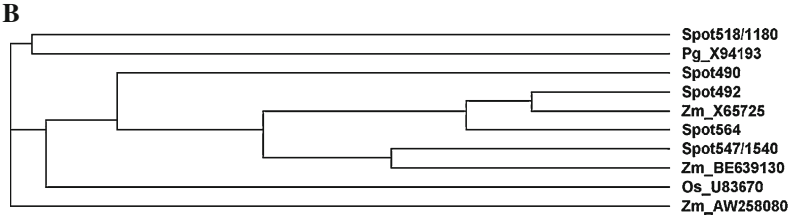

Fig. 2 Peptide sequence alignment of spots 490, 492, 547, 564, 518, and 1540 from embryo and spot 1180 from endosperm with small heat-shock proteins from maize and other plant species (**a**) and a cladogram showing their relative similarities (**b**). X65725 is a 17.2-kDa heat shock protein (17.2 HSP) from *Zea mays* (Zm); X94193 is a 17.9-kDa heat shock protein from pearl millet (*Pennisetum glaucum*, Pg); U83670 is an 18-kDa heat shock protein from *Oryza sativa* (Os); AW258080 and BE639130 are ESTs from *Zea mays*. The underlined "V" indicates the difference between spot 564 and spot 492. The amino acid residues that are identical or highly conserved among the species are indicated with "*" and ".", respectively

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Zm_ACF83600       VAVGNEAQGDDTRSLLPAMRN-LDAALARAGFFPGIKCSTS-VRFDVVANSFPPSSGSFA 178
Spot842           VAVGNEVQGDDTR------------------------------------------------ 14
Sb_EES04197       IAVGNEVQGGATQSILPAIRN-LDAALARAG-LSAIKCSTS-VRFDVIANSYPPSSGSFA 175
Ta_AAY88778       IAAGNEVQGGDTQSIVPAMRN-LNAVLSAAG-LSAIKVSTS-IRFDAVANSFPPSAGVFA 173
Hv_P15737         IAAGNEVQGGATQSILPAMRN-LNAALSAAG-LGAIKVSTS-IRFDEVANSFPPSAGVFK 173
Sc_AM181314       IAAGNEVLGGATQSIVPAMRN-LNAALSAAG-LGAIKVSTS-IRFDAVANSFPPSAGVFA 145
Sc_CAJ58511       IAAGNEVLGGATQSIVPAMRRPQRGPLRRRP-RRHQGVHLDPVRRGGQHHSHPPPA--CS 116
Os_EAY77172       IAVGNEVTGDDTGNILPAMKN-LNAALAAAG-LGGVGVSTS-VSQGVIANSYPPSNGVFN 169
                  :*.***. *. *

Zm_ACF83600       QGYMADVARYLAGTGAPLLANVYPYFAYRDNPRDISLGYATFQPGTTVRDNGNGLNYNNL 238
Spot842           ----------------------------------DISLGYATFQPGTTVR------------ 30
Sb_EES04197       QGYMADVARYLAGTGAPLLVNVYPYFSYRDNPRDISLGYATFQPGTTVRDNGNGLTYTNL 235
Ta_AAY88778       QSYMTDVARLLASTGAPLLANVYPYFAYRDNPRDISLNYATFQPGTTVRDQNNGLTYTSL 233
Hv_P15737         NAYMTDVARLLASTGAPLLANVYPYFAYRDNPGSISLNYATFQPGTTVRDQNNGLTYTSL 233
Sc_AM181314       QSYMTDVARLLASTGAPLLANVYPYFAYRDNPRDISLNYATFQPGTTVRDQNNGLTYTCL 205
Sc_CAJ58511       RAYMTDVARHLASTGAPLLANVYPLPSYRDNPRDISLNYATFQPGTTVRDQNNGLTYTCL 176
Os_EAY77172       DDYMFDIVEYLASTGAPLLVNVYPYFAYVGDTKDISLNYATFQPGTTVTDDGSGLIYTSL 229
                                      .***.**********
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Fig. 3 Peptide sequence homology analysis of spot 842 with β-1,3-glucanases from wheat, barley, rice, and rye. ACF83600, an unknown protein from *Zea mays* (Zm); EES04197, a hypothetical protein from *Sorghum bicolor* (Sb); AAY88778, β-1,3-glucanase from *Triticum aestivum* (Ta); P15737, a β-1,3-endoglucanase from *Hordeum vulgare* (Hv); AM181314, glucan endo-1,3-β-D-glucosidase precursor; gluna-2 gene from *Secale cereale* (Sc) (rye); CAJ58511, glucan endo-1,3-β-D-glucosidase from *Secale cereal*; and EAY77172, a hypothetical protein from *Oryza sativa* (Os) (*indica* group)

Van Inghelandt et al. 2010), their application in breeding for aflatoxin resistance is limited due to difficulty in establishing their consistent association with quantitative trait loci (QTL). A comparative proteomics approach was used in previous studies in an effort to identify RAPs that may have potential use as markers (Chen et al. 2002, 2007). The first approach taken involved the analysis of a number of unrelated aflatoxin-resistant and -susceptible lines, and the construction of composite protein profiles of the lines in order to eliminate the effect of genetic background differences on the identification of RAPs. This required significantly more time to identify RAPs than did the use of closely-related lines that differed in resistance to toxin accumulation. The kernels used in the present study were grown in Saminaka in Nigeria, which receives sufficient precipitation every year and where pressure from major lowland diseases is minimal. Therefore, the observed protein differences between the lines in our proteomic comparisons would be expected to be due to differences at the constitutive protein level. However, the possibility cannot be ruled out that the differences might be further enhanced by exposure to pathogens (including *A. flavus*) during plant development. The strength of the present study is that at least three pairs of closely-related lines were analyzed to identify constitutive protein differences between resistant and susceptible lines. Most of the differentially expressed embryo constitutive proteins identified in the present study were up- or down-regulated in up to four pairs, but no single embryo protein spot was commonly up- or down-regulated in the resistant lines of all pairs, indicating the possible presence of different resistance mechanisms.

Results of the present study support previous findings (Chen et al. 2002, 2007), which also identified storage, stress-related, and antifungal proteins as RAPs. A large number of previously identified stress-related proteins, such as HSPs, showed differential expression between resistant and susceptible closely-related lines in the present study, indicating their importance in resistance to pathogen attack, which is regarded as a unique stress (Wan et al. 2002). In response to such stress, plants not only induce specific antifungal proteins, but also up-regulate general stress-related proteins, such as plant small HSPs, that function as molecular chaperones to enhance kernel stress tolerance (Sun et al. 2002). In addition to small HSPs, several other previously identified stress-related RAPs were also differentially expressed in the present study, including glyoxalase, cold regulated protein and cupin-domain-containing proteins in the embryo as well as globulin-2, superoxide dismutase, peroxiredoxin and late-embryogenesis-abundant proteins in the endosperm. The potential for involvement of some of these proteins in maize stress tolerance or resistance to *A. flavus* has been demonstrated. For example, the cold regulated protein, ZmCORp, has been found to inhibit germination of *A. flavus* conidia and mycelial growth (Baker et al. 2009), while glyoxalase reduces methylglyoxal, a potent cytotoxic compound spontaneously produced in all organisms and a transcription inducer of the aflatoxin pathway regulatory gene *AflR* (Chen et al. 2004). Superoxide dismutase and peroxiredoxin (PER1), an antioxidant with peroxidase activity (Chen et al. 2007), may also enhance oxidative stress tolerance in maize. The possible involvement of cupin-domain-containing proteins in maize aflatoxin resistance is not clear, although such proteins have been shown to function as enzymes or transcription factors (Dunwell et al. 2004). In light of the positive correlation between drought stress and aflatoxin accumulation (Payne 1998), the identification of these stress-related proteins in the present study further highlights their importance in maize resistance to *A. flavus* infection.

Several antifungal proteins also showed differential expression between resistant and susceptible closely-related lines, such as the previously reported 14-kDa trypsin inhibitor and the 22-kDa zeamatin/trypsin inhibitor protein. Both inhibit *A. flavus* growth (Huynh et al. 1992; Guo et al. 1997; Chen et al. 1998). The present study also uncovered a new basic β-1,3-glucanase with an estimated pI of 9.90 and molecular mass of 33.5 kDa. This is different from the previously reported maize acidic (AAA74320, AAT42176, Q9ZT66, ACG32824) (Wu et al. 1994; Thomas et al. 2000; Swigonova et al. 2004; Alexandrov et al. 2009) or basic glucanases (AY344632, Suen et al. 2003). It could be the basic PRm Ba1 protein with intracellular glucanase activity reported by Nasser et al. (1990), based on its size and pI.

In addition, a new category of regulatory proteins was identified as RAPs, which includes a putative lipid transfer protein and a eukaryotic translation initiation factor 5A. This was possibly the result of

using closely-related lines, which allowed us to lower the threshold from 2-fold or higher in earlier studies to 1.5-fold due to increased genetic background similarities and reduced technical variations in proteomic analysis. Plant lipid transfer proteins are ubiquitous lipid-binding proteins involved in various stress responses (Wang et al. 2009) and the translation initiation factor is also known to play a crucial role in plant growth and development (Feng et al. 2007). This group of constitutively expressed proteins may play a role in regulating the host plant expression of downstream RAPs in response to *A. flavus* infection. Further characterization may pinpoint the roles of these proteins in maize resistance to *A. flavus* infection and aflatoxin contamination.

The present study identified the presence of a second type of glyoxalase in maize embryo, represented by spot 546. This protein was missing in pair no. 1, but was differentially expressed in three other pairs. This family of proteins has a size of about 150 amino acids, which is different from spot 384 and the previously reported long-type maize glyoxalase I protein (GLX-I) of about 290 amino acids (Chen et al. 2004). The higher expression of the long-type GLX-I (spot 384) in the resistant line of pair no. 1 may functionally complement the absence of short type GLX (spot 546).

Some of the RAP genes have been mapped to chromosome regions containing major QTL found in earlier studies to contribute to maize aflatoxin resistance. For example, TI and an embryo-specific protein (spot 337) are located at bin 2.06 and bin 4.06, respectively. The same chromosome regions were found to contain two major QTL that each accounted for 7–18% of phenotypic variation (Brooks et al. 2005). Two other RAP genes were located at bin 1.03 (HSP17.2/GLX) and bin 3.05 (glucanase), where two minor loci were also found to contribute significantly to phenotypic variation in the same study (Brooks et al. 2005). Paul et al. (2003) also found a QTL at bin 3.05–6 to contribute to maize aflatoxin resistance. Two major QTL located at bin 3.06 and bin 4.06 were also found in a recent mapping study using a population developed from Mp715 to T173 (Warburton et al. 2010). Several other RAP genes have also been located on chromosome regions that were previously reported to contain major QTL, such as glucose dehydrogenase at bin 2.08 (Busboom and White 2004). The association of some of the identified RAPs with reported major QTL linked to aflatoxin resistance may help us to narrow down candidate genes that should be tested as markers.

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